THE JOURNAL OF GENE MEDICINE
J Gene Med 2001; 3: 59–71.

RESEARCH ARTICLE

Optimised helper virus-free production of high-quality adeno-associated virus vectors

Lila Drittanti*†
Christine Jenny
Karine Poulard
Anne Samba
Peggy Manceau
Nestor Soria†
Nathalie Vincent
Olivier Danos
Manuel Vega†

Genethon III, CNRS URA 1923, 1 bis rue de l'internationale, 91002 Evry, France

*Correspondence to: L. Drittanti, Genethon III, CNRS URA 1923, 1 bis rue de l'internationale, 91002 Evry, France.
E-mail: drittant@genethon.fr

†Current address: Nautilus Biotech, Evry, France.

Received: 27 June 2000
Revised: 8 September 2000
Accepted: 28 September 2000
Published online: 9 October 2000

Abstract

Background Clinical development of adeno-associated virus (AAV) requires standardised, safe, efficient and scalable procedures for the manufacture of the rAAV vector, including production, purification and testing. Several strategies have been reported for the approach to the manufacturing problem. We report a helper virus-free process that produces high quality rAAV stocks.

Methods rAAV were produced by triple transfection, a helper virus-free process. After lysis of the cells in the presence of nuclease, the rAAV produced were purified by HPLC through two ion-exchange columns in tandem followed by dialysis. rAAV stocks were thoroughly characterised for biological activity and for the presence of residual contaminants. The titer of infectious particles and of rep+ particles was determined by dRA assay. Contaminating DNA and RNA were determined by fluorescent dye binding and real-time PCR. The protein content of the rAAV stocks was characterised by SDS-PAGE, ELISA test, Western blot and specific enzymatic assays for putative residual contaminating protein. The *in vivo* biological activity of the stocks was evaluated in mouse muscle.

Results rAAV stocks obtained following this procedure elicit: $2–5 \times 10^{12}$ pp/ml; $3–6 \times 10^{10}$ ip/ml; $<10^3$ rep+ particles/ml; <0.3 mUeq/ml of residual benzonase activity; non-detectable Ad or β-galactosidase proteins; <35 pg/ml of cellular genomic DNA; *in vivo* expression in mouse muscle without any immune reaction detected.

Conclusions This work demonstrates the possibility of producing purified high-quality rAAV free of helper virus. The procedure described in this paper is easily adaptable for large-scale production of clinical rAAV vectors. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords adeno-associated virus vectors; vector production; vector purification

Introduction

Adeno-associated virus (AAV), a single-stranded DNA virus from the parvovirus family [1], has become one of the most promising vectors for human gene therapy for several reasons. AAV has not been implicated as the causative agent for any human disease. Studies to determine the status of the rAAV following *in vivo* transduction indicate that the vector may be integrated at random sites in the liver, haemopoietic cells and neurones as head-to-tail concatameric arrays [2–4]. In lung tissue and in skeletal muscle, nevertheless,

the rAAV genome has been detected in the episomal form [5–7]. rAAV can efficiently transduce both growing and post-mitotic cells when administered directly to tissues and organs *in vivo*. In addition, rAAV-mediated gene transfer has been reported to result in only moderate and transient immune responses [8]. Moreover, rAAV vectors have been used to transfer a number of different genes into several target tissues in animal models, showing the potential of rAAV for the treatment of human disease [9–18]. Long-term transgene expression has been shown *in vivo* after rAAV transduction [10,19]. Not surprisingly, these observations have moved the field of rAAV vector development to the fore of gene therapy delivery systems. Two clinical reports have been published for rAAV indicating the absence of toxicity or immune response [20] and the lasting transgene expression and biological activity of the therapeutic protein [21].

rAAV requires both host cell factors and co-infection with Ad or herpes viruses for a productive infection cycle. Wild-type (wt) AAV replicates to high titers in the cell, however productivity of rAAV (in terms of particles per cell) is consistently about two orders of magnitude lower in comparison to wt AAV. Thus, the use of rAAV in gene therapy has been limited by the availability of high titer and pure vector stocks. Several approaches to vector production have been reported including the use of stable producer cell lines [22,23] and the transient co-transfection of vector and helper plasmids [24]. In both cases subsequent infection with Ad is required. However, Ad helper genes are expressed as a part of the replication cycle of the infecting Ad itself, leading to the production of new Ad particles. Another approach for rAAV production has been the use of a helper plasmid to provide the essential adenovirus genes [25–27]. In this system infection with helper virus is not necessary and, consequently, the rAAV produced are expected to be free of viral contaminants. For this reason we adopted this triple-transfection system as the starting procedure in our optimisation of rAAV manufacturing.

More recently, herpes virus constructs (herpes simplex virus type 1 amplicon and vector) harbouring the AAV rep and cap genes have been used for rAAV production [28], indicating the possibility of high outputs of up to 1000 infectious rAAV particles per cell [28,29]. However, current as well as new processes for the purification of rAAV must be evaluated to eliminate all possibilities of contamination with herpes virus.

The quality of rAAV vector stocks needs to be improved and properly assessed by several criteria in order to develop a manufacturing process suitable for the production of high-quality rAAV stocks to be used in preclinical studies and ultimately in clinical trials.

To address these issues we have optimised an Ad helper- and wt AAV-free production procedure for rAAV. In addition, we have developed a simple, rapid, reproducible, effective and scalable downstream process that yields high-purity and high-quality rAAV stocks that are biologically active *in vivo*.

Materials and methods

Cells, plasmids and viruses

293 human embryo kidney (HEK) cells, obtained from American Type Culture Collection (ATCC), were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose (DMEM; Gibco-BRL, Cergy Pontoise, France) 10% foetal bovine serum (FBS; Hyclone/PerbioScience, Bezones, France), 1% penicillin and streptomycin (Gibco-BRL). Every 20 passages after thawing, cultures were re-started from a new ampoule. Cells were tested for sterility and mycoplasma.

HeLa and HeLa rep-cap32 cells were obtained from ATCC and from Anna Salvetti (CHU, Nantes), respectively. HeLa rep-cap32 cells express the wt AAV rep and cap genes. HeLa and HeLa rep-cap32 cells were maintained in DMEM supplemented with 10% FBS (Hyclone) and 1% penicillin and streptomycin (Gibco-BRL).

pXX6 (Ad helper) [25], pBHG10 (Ad helper), pACG2 (AAV helper) [30], pRC4 (AAV helper) and pAAV-CMV(nls)lacZ (rAAV vector) [31] plasmids were prepared on DH5α *Escherichia coli* and purified by PEG (polyethylene glycol) according to standard procedures. Plasmid batches were pooled-out to constitute homogeneous plasmid working stocks used for rAAV manufacturing. Release criteria for the plasmid working stocks included restriction analysis, assessment of contaminating RNA and genomic DNA, OD_{260}/OD_{280} ratio, OD_{320} and content of supercoiled plasmid DNA.

Drs R. Jude Samulski and A. Salvetti kindly provided starting samples of pXX6 (R.J.S.), pACG2 (R.J.S.) and pAAV-CMV(nls)lacZ (A.S.). pBHG10 was obtained from Microbix Biosystems Inc., (Ontario, Canada) pRC4 was engineered by subcloning the XbaI-XbaI fragment from pACG2, containing the rep and cap genes, into the MCS of the pBS-KSII+. pRC4 does not contain the Ad-ITRs present in pACG2.

Ad dl324 adenovirus stock, originally provided by E. Kremer, was produced according to standard procedures. Wild-type adenovirus type 5 stocks were kindly provided by P. Moullier.

rAAV production

rAAV were produced by triple transfection on 293 HEK cells. Cells (1×10^9 aliqouts) were seeded into 30 triple flasks and cultured for 24 h before transfection. Transfection was performed on ~70% confluent cells.

25 kDa PEI (polyethyleneimine, Sigma-Aldrich, St. Quentine Fallavier, France) was used for the triple transfection step. Equimolar amounts of the three plasmids 450 μg Ad helper plasmid (either pXX6 or pBHG10), 275 μg AAV helper plasmid (either pACG2 or pRC4) and 275 μg (AAV plasmid vector) were mixed with 10 mM PEI by gently shaking. The mixture was then added to the culture medium on the cells. One milligram of total plasmid was added to every 2×10^9 cells in

culture. Cell culture was continued as described above except with 1% instead of 10% FBS. Six hours after transfection the medium composition was modified to 3 g/l glucose and 5% FBS by the addition of fresh culture medium (containing 1 g/l glucose and 10% FBS).

Alternatively to the triple transfection, when indicated in the text, rAAV was produced by double transfection (using the AAV helper plasmid and the AAV plasmid vector) followed, 6 h later, by infection with helper Ad (Ad dl324, MOI of 10). Transfection and cell culture conditions were as described above.

Alternatively to the use of PEI, triple transfection was performed by the $CaPO_4$ co-precipitation method. Equimolar amounts of the three plasmids and 1 mg total plasmid were added to 2×10^9 cells in culture. Cell culture was continued in the presence of 1% FBS. Six hours after transfection, the culture medium was replaced with fresh medium containing 3 g/l glucose and 5% FBS.

Forty-eight hours after transfection, the culture medium was replaced with 200 ml PBS. Cells were detached by shaking, centrifuged (15 min at 1200 *g*) and resuspended in 100 ml lysis buffer (50 mM HEPES, pH 7.4; 150 mM NaCl; 1 mM $MgCl_2$; 1 mM $CaCl_2$). After four cycles of freeze–thawing in the lysis buffer, 250 U benzonase (Merck, Nogeut sur Marne, France) were added. Lysate viscosity was reduced by incubation at 37°C for 30 min. Cellular debris was eliminated from the lysates by centrifugation at 10 000 *g* for 30 min at 4°C. The supernatant was filtered through a 0.45 µm pore size disc filter (Pall-Gelman, St. Germain eu Laye, France), giving rise to the 'semi-purified stock' (SS), and recovered for further downstream processing. From a culture of 1×10^9 cells, 60 ml SS were routinely obtained. The cell lysis step, from the addition of the lysis buffer to the recovery of the SS, was performed at room temperature. The SS was stored at −20°C until required for further use.

Downstream processing

The SS were diluted up to 200 ml final volume in PBS and then subjected to diafiltration in a Filtron system (Pall-Gelman) using Omega open-channel membranes (300 kDa pore size). Diafiltration was continued until 40 ml final volume was obtained. After addition of 160 ml PBS to the diafiltrate, diafiltration was repeated. The complete operation (diafiltration, completion to 200 ml, diafiltration) was repeated twice. The 40 ml obtained from the third diafiltration were directly loaded onto the HPLC columns in tandem. A Biocad 700E workstation for perfusion chromatography system (PerSeptive Biosystems, Perkin Elmer, Les Ulis, France) was used in conjunction with a Poros HQ anionic exchanger column (50 µm bead size, 20 ml bed volume) in tandem with a Poros SP cationic exchanger column (20 µm bead size, 1.7 ml bed volume). The HQ and SP columns were attached in a dual-column configuration. Columns were equilibrated with 100 mM HEPES, pH 8.0. The 40 ml diafiltrates were applied at a flow rate of 2 ml/min at room temperature. After sample loading, the in-tandem columns were washed with 200 ml 100 mM HEPES, pH 8.0. rAAV were retained by the SP column while ~50% of the contaminating proteins present in the SS were retained by the HQ column. The SP column was directly eluted by means of a 0–1 M NaCl linear gradient in 100 mM HEPES, pH 8.0. rAAV were recovered at 28–30 mS (300 mM NaCl). A continuous register of OD_{260}, OD_{280} and conductivity was performed, and 0.6 ml fractions were collected.

All steps from ultrafiltration to the collection of the eluate from the second HPLC column routinely took about 5 h at room temperature.

Fractions eluted from the second column were analysed by dot blot hybridisation (rAAV genome), by a functional test for transgene activity (β-galactosidase) and by immunoblotting (anti-AAV capsid protein). Fractions containing rAAV particles were pooled and further processed. Routinely, five fractions were pooled together, corresponding to a volume of 3 ml recovered from 1×10^9 initial cells/2.5 l of initial culture.

The 3 ml HPLC pooled fractions were dialysed against 2 l Ringer-lactate buffer at 4°C for 18 h, with three changes of buffer. The final dialysate was recovered, filtered through a 0.2 µm pore size disc filter (Pall-Gelman) and stored at −80°C. The final preparation was routinely characterised as described below.

Physical particles

Samples of rAAV (10 µl) were incubated with 10 U DNAse I (Gibco-BRL) at 37°C for 60 min and then with 100 µg proteinase K. After phenol : chloroform extraction, viral DNA was precipitated with ethanol in the presence of 40 µg glycogen, washed twice with 70% ethanol and resuspended in 400 µl 0.4 M NaOH, 10 mM EDTA. Samples were dotted onto nylon membranes (Pall-Gelman), washed once with 400 µl 2 × SSC, pre-hybridised for 60 min at 65° in hybridisation buffer (0.5 M sodium phosphate buffer pH 7.4, 7% SDS, 1 mM EDTA, 100 µg salmon sperm DNA) and finally hybridised overnight with a specific lacZ probe. Dot intensity was measured using a Storm 840 System (Molecular Devices, Amersham, UK) and quantitated by comparison with a standard curve obtained using the rAAV plasmid.

rAAV transducing units

The titer of lacZ + forming units (Lfu) was determined on 293 HEK cells. Twenty-four hours before infection, cells were plated at a density of 1×10^5 cells/well in 24-well plates. Serial dilutions of the rAAV preparation were made between 1×10^{-2} and 1×10^{-5} and used for co-infection of the 293 HEK cells together with Adl324 adenovirus (MOI of 10). Twenty-four hours after infection, X-gal staining was performed according to standard procedures.

rAAV infectious particles

The titers of rAAV vector infectious particles were determined on HeLa rep-cap32 cells using our standard dRA (serial dilution replication assay) test described elsewhere [32]. Cells were plated 24 h before infection at a density of 2×10^4 cells in 96-well plates. Serial dilutions of the rAAV preparation were made between 1×10^{-2} and 1×10^{-12} and used for co-infection of the HeLa rep-cap32 cells together with type 5 wt Ad (MOI of 100). Cells were lysed 24 h after infection with 50 µl lysis solution (0.8 M NaOH, 10 mM EDTA, 0.5 ng salmon sperm DNA) (65°C, 30 min) by gentle shaking. Lysates were loaded onto positively charged nylon membranes (Pall-Gelman) and washed with 400 µl 0.8 M NaOH, 10 mM EDTA. Membranes were rinsed once with 2 × SSC solution (room temperature, 15 min). Controls included mock cells, cells infected only with rAAV (1 µl undiluted preparation) and cells infected only with wt Ad (MOI of 100). Membranes were pre-hybridised in hybridisation buffer (65°C, 1 h) and hybridised with a lacZ specific probe (overnight, 65°C). Radioactivity was quantified in a Storm 840 (Molecular Devices). Hybridisation controls included purified probe DNA as the positive control and salmon sperm DNA and lysis solution as negative controls.

rep+ AAV

The titers of rep+ AAV particles were determined on HeLa cells using the serial dilution dRA assay [32]. Cells were plated 24 h before infection at a density of 2×10^4 cells in 96-well plates. Serial dilutions of the rAAV preparation were made between 1×10^{-2} and 1×10^{-9} and used for co-infection of the HeLa cells together with type 5 wt Ad (MOI of 100). Cells were lysed 24 h after infection. Lysis and further processing were performed as described in the previous section for the titration of infectious particles.

AAV capsid proteins

Capsid proteins, empty and full virion-capsid, were quantified by a specific ELISA test kit (Progen/Tebu, Le Perray-eu-Yvelines, France). The number of total (full plus empty) capsids (P) was determined from the results obtained with the A20 monoclonal antibody. Assuming the protein molecular mass of an intact AAV-2 capsid to be 4×10^6 Da, then 1 µg capsid proteins would equal 1.5×10^{11} rAAV virions. The number of full capsids (pp) was derived from the number of rAAV genomes obtained by dot blot hybridisation. Finally, the number of empty capsids was calculated as the difference between P and pp.

VP1, VP2 and VP3 proteins were detected by Western blot analysis as follows. 10 µl HPLC fractions (positive for rAAV dot hybridisation) were subjected to SDS-PAGE electrophoresis on 8% polyacrylamide gels in 192 mM glycine, 25 mM TRIS pH 8.3, 0.1% SDS and electro-blotted onto PVDF membranes (Biorad, Ivry sur Seine, France) using 39 mM glycine, 48 mM TRIS pH 8.3, 0.04% SDS, 20% ethanol buffer. Gels were calibrated using Kaleidoscope molecular weight standards (Biorad).

Membranes were blocked with 1% BSA (Biorad) in TBS buffer (RT, 60 min) and incubated (RT, 60 min) with a 1/10 dilution of the commercial mouse antibody against VP1, VP2 and VP3 AAV proteins (clone B1, Progen/Tebu). Membranes were then incubated with an anti-mouse goat antibody conjugated with alkaline phosphatase and visualised using the Biorad kit.

Residual protein contamination

The total protein content in the rAAV samples was determined by Bradford protein assay (Biorad). Samples included crude lysates (1–10 µl), HPLC fractions (10 µl) and final rAAV stock.

Individual proteins were separated by SDS-PAGE electrophoresis on 8% polyacrylamide gels in 192 mM glycine, 25 mM TRIS pH 8.3 and detected with a silver staining kit (Sigma-Aldrich). Gels were calibrated using the high-range molecular weight standard from Biorad.

Residual benzonase activity

Ten microliters of either rAAV FS or standard DNA solution (1 µg/µl pXX6) plus 15 µl benzonase buffer (62.5 mM TRIS pH 8, 62.5 mM MgCl, 1.25 mg/ml BSA) were mixed together in a final volume of 25 µl and incubated for 16 h at 37°C. The reaction was stopped by the addition of 3 µl loading buffer. The digested plasmid DNA was analysed on 1% agarose gels. The migration distance (the distance from the origin to the position of the plasmid product band) was measured and used to estimate the benzonase activity content from a standard curve. The standard curve was plotted each time using a fresh benzonase stock solution (250 U/µl stored at −20°C). Serial dilutions of the benzonase stock were incubated in parallel with the rAAV final stock samples in the presence of the same amount of substrate DNA and run in the same agarose gel. The migration distance of the plasmid product (mm) was plotted against the corresponding benzonase activity (number of units) present in the calibration sample. Using our protocol, as little as 0.3 mU benzonase in a 10 µl sample can be measured.

As our benzonase test was performed over 16 h, instead of the 30 min used for the definition of 1 unit (U), we define here a benzonase equivalent unit as the amount of benzonase that exhibits an activity equivalent to 1 U, when tested in a 16 h assay.

Residual RNA and DNA

Residual RNA and double-stranded (ds)DNA contamination were determined in the rAAV SS and FS. The presence of RNA was assessed on 5 µl (SS) or 10–50 µl (FS) by the Ribo-Green quantification kit (Molecular Probes, Amersham Pharmacia Biotech, Saday, France) that permits the detection of as little as 200 pg RNA. The

presence of DNA was assessed on 5 µl (SS) or 10–50 µl (FS) by the Pico-Green quantification kit (Molecular Probes) that permits detection of as little as 25 pg DNA.

The presence of contaminating cellular genomic DNA was evaluated by real-time PCR [33] using a PE-Applied Biosystems Prism 7700 (Perkin Elmer). The primers and fluorescence probe sets corresponding to the albumin gene and ALU sequences were as follows:
ALB1 primers 5′-GCTGTCATCTCTTGTGGGCTGT-3′ (0.2 µM) and ALB2 primer 5′-ACTCATGGGAGCTGCTGG TTC-3′ (0.2 µM); probe 5′-VIC CCTGTCATGCCCACA CAAATCTCCCC-3′ (0.1 µM).
ALU1 primer 5′-TTGCAGTAAGCCGAGATCCC-3′ (0.2 µM) and ALU2 primer 5′-TTGAGACGGAGTCTCGCTCTG-3′; probe 5′-FAM CTGCACTCCAGCCTGGGCGAC-3′ (0.1 µM).

The copy number of the albumin gene was evaluated in sample aliquots of 10 µl. Calibration was performed with isolated cellular genomic DNA (293 HEK cells). A rAAV stock obtained by lysis in the presence of DNAseI and RNAse instead of benzonase was used as a secondary positive control.

In vivo activity

Four-week-old Balb-c mice (Charles River, St. Germain sur L'Arbresle, France) were injected into the *tibialis anterior* muscle with 1×10^7 rAAV infectious particles suspended in Ringer solution. At various time points after injection mice were killed and injected muscle tissue was harvested and immediately frozen in liquid nitrogen. Cryostat sectioning of the tissue was performed at 10 µm thickness with a Leica 3050 microtome. Sections were fixed and stained successively with X-gal and hematoxylin using standard procedures [34].

Results

This work describes the three steps undertaken to define the manufacture of rAAV stocks: (1) optimisation of production conditions, (2) up-scaling of production and downstream processing and (3) characterisation of the stocks produced.

Optimisation of rAAV production

In order to optimise the rAAV production process, in terms of productivity, content of rep+/cap+ particles, simplicity and reproducibility, the following parameters were evaluated: helper Ad, helper AAV, transfection procedure and time of harvesting.

Double transfection followed by infection with a helper adenovirus (Adl324) was compared to triple transfection for the production of rAAV. The Ad helper plasmids used for the triple transfection retained either all viral genes except E1 (pBHG-10) or only the set of genes necessary for AAV replication (pXX6) [25]. As seen in Table 1, there is no significant difference in rAAV productivity (either in physical or in infectious particles) when the pXX6 Ad helper plasmid is used instead of the infectious Ad dl324. Moreover, pXX6 plasmid was more productive than pBHG10 which, in addition, led to a higher ratio of rAAV empty capsids/physical particles. Therefore, all subsequent rAAV production was performed using triple transfection and the pXX6 plasmid as Ad helper.

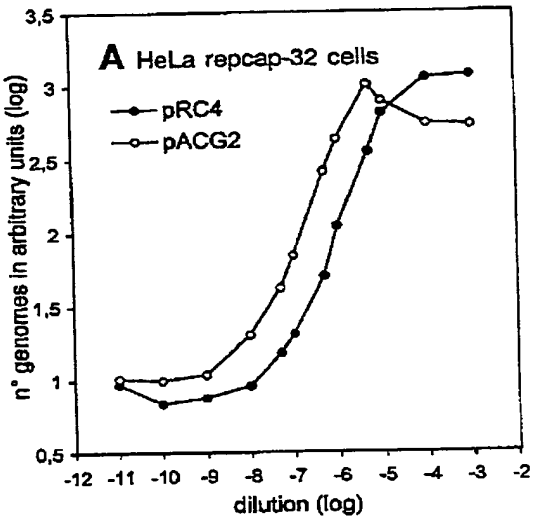
Two different AAV helper plasmids [pACG2 (containing the Ad-ITR sequences) or pRC4 (Ad-ITR sequences deleted)] were compared for the triple transfection step As shown in Figure 1, deletion of Ad-ITR led to at least a 1000-fold decrease in the content of rep+ particles produced (Figure 1B), while the titer of rAAV obtained remained unchanged (Figure 1A). The generation of infectious replication competent progeny requires the presence of both rep and cap genes. The rep+ particles shown in Figure 1 do not necessarily represent replication-competent AAV. In order to assess the level of AAV particles able to generate rep+/cap+ progeny, serial infection experiments were performed. rep and cap DNA was amplified and quantitated by standard PCR on cell lysates obtained after a number of infection cycles with infected cell lysates. The first cycle consisted of infecting 4×10^{-5} HeLa cells, plated 24 h prior to infection in the presence or the absence of type 5 wt Ad (MOI of 50). Samples were used for both re-infection of fresh HeLa cells and PCR analysis for the presence of rep and cap genes. Re-infection was repeated five times to complete six infection cycles in order to allow for the amplification of the rep+/cap+ particles potentially present. As expected [31], pACG2 generated significantly higher

Table 1. Comparison of plasmid- and virus-mediated adeno-helper function for the production of rAAV

	rAAV preparation					
Ad helper	pp/ml	ip/ml	pp/ip	pp/cell	P/ml	P/pp
pXX6	$6.1 \pm 0.2 \times 10^{11}$	$7.5 \pm 1.0 \times 10^{9}$	70–100	$10\,100 \pm 1500$	$2.0 \pm 0.3 \times 10^{12}$	4.25–2.85
pBHG10	$7.0 \pm 1.0 \times 10^{10}$	$6.0 \pm 1.1 \times 10^{8}$	120–130	3000 ± 600	$5.0 \pm 1.0 \times 10^{11}$	6.95–7.50
Adl324	$4.6 \pm 0.2 \times 10^{11}$	$4.2 \pm 0.9 \times 10^{8}$	100–120	7600 ± 1200	$3.0 \pm 0.4 \times 10^{12}$	5.70–7.65

rAAV were produced by triple transfection into three flasks using either plasmids (pXX6 or pBHG-10) or by transfection/infection with adenovirus (Ad dl 324) as described in Materials and methods. The rAAV preparations obtained were titered for physical particles (pp) by dot blot assays, for infectious particles (ip) by dRA assays and for total capsid (P) by ELISA as described in Materials and methods ($n = 3$).

amounts of rep+/cap+ particles (20–50 ng and higher than 50 ng DNA each gene after the first and sixth cycles, respectively) than pRC4 (5–50 pg cap DNA and less than 5 pg rep DNA in the first cycle and 5–50 pg each gene after the sixth cycle). According to these data and to Figure 1B, deletion of the Ad-ITR in the pRC4 plasmid led to at least a 1000-fold decrease in the level of rep+/cap+ particles.



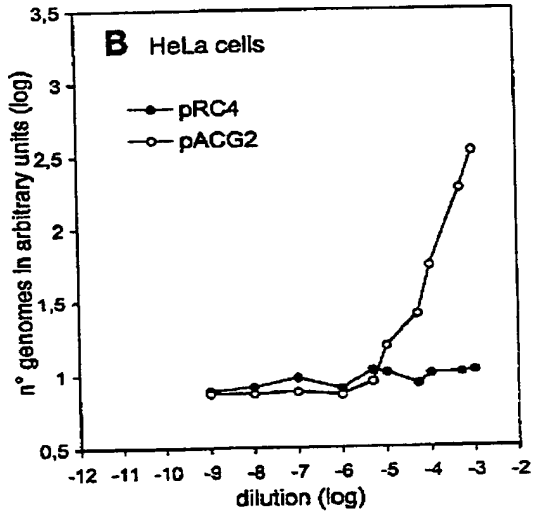


Figure 1. Comparison of pACG2 and pRC4 for the production of rAAV and contaminating rep+AAV. rAAV were produced by triple transfection into two flasks using either pACG2 or pRC4 as described in Materials and methods. The rAAV preparations obtained were titered by dRA assays [HeLa RC 32 (A) or HeLa (B) cells were infected with serial dilutions of the rAAV preparation in the presence of wt adenovirus to allow for rAAV replication]. Twenty-four hours later the number of replicating genomes was determined by dot blot and is indicated in the figure as a function of rAAV dilution. Titers are calculated from the second order polynomial function [f(x)=a1+a2 th(a3 x+a4), where x is measured by the log (1000/dilution) and f(x) by the log (1/intensity)] by non-linear regression fitting the experimental data. The point where the polynomial curve reaches its minimum was considered to be the titer of the rAAV preparation. Titers obtained were (A) infectious particles (ip) 2×109 ip/ml using either plasmid for the transfection step; (B) rep+ particles were 1×106 ip/ml for pACG2 and non-detectable for pRC4

Two DNA transfection methods were tested for the triple transfection step for the comparison of rAAV productivity. Plasmid DNA was transfected into 293 HEK cells either complexed with PEI or precipitated with $CaPO_4$. Cells (7×10^7, two triple flasks) were transfected with a mixture of pXX6, pACG2 and pAAV-CMV(nls)lacZ as described in Materials and methods. The transfection efficiency measured as the percentage of β-gal+ cells (lacZ forming units, Lfu) was in the range 50–60% in seven independent experiments for either method. rAAV productivity was expressed as the number of physical particles (pp) and the pp/Lfu ratio. In Figure 2 we show that both methods were equally efficient for rAAV pp production, although PEI leads to lower and more reproducible pp/Lfu ratios. In addition, the use of PEI greatly simplifies the transfection procedure. Therefore transfection with PEI-DNA complexes was adopted as the methodology for further rAAV production runs.

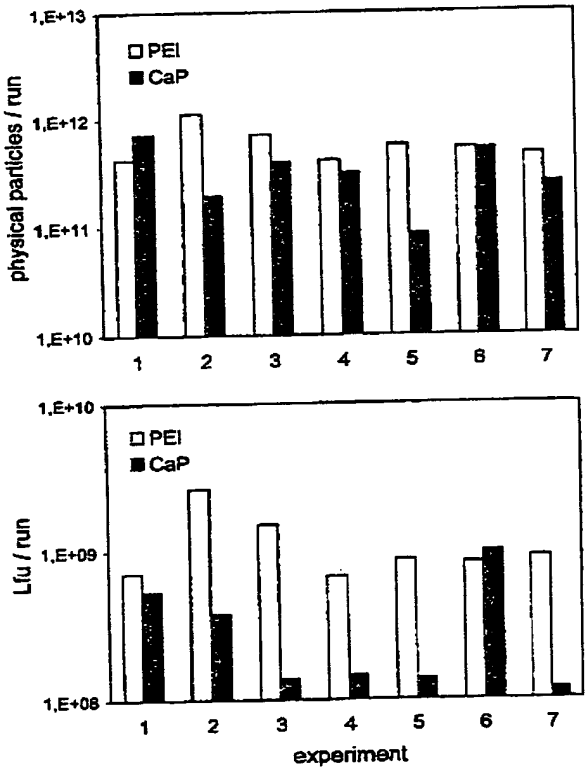


Figure 2. Comparison of PEI and CaP at the transfection step for the production of rAAV. rAAV were produced by triple transfection into two flasks using either PEI or $CaPO_4$ as described in Materials and methods. (A) rAAV titers of physical particles (pp) by dot blot hybridisation and (B) transducing units (Lfu) obtained by X-gal staining of infecting cells. Data obtained from seven independent experiments are shown

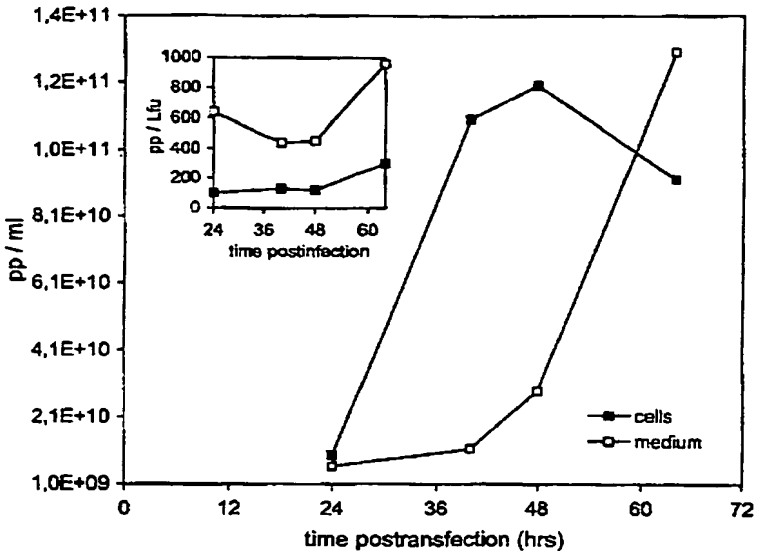


Figure 3. rAAV recovery over time post-transfection. rAAV were produced by triple transfection into two flasks using PEI as described in Materials and methods. rAAV produced were harvested at increasing time points (24, 40, 48 and 66 h) after transfection. The number of physical particles (pp) as well as transducing units (Lfu) produced were determined as described in Materials and methods. Titers in pp/ml and the ratio pp/Lfu are shown

In order to identify the optimal time point for the harvesting of the rAAV before significant cell lysis and virus release into the culture medium occurs a kinetic study was undertaken. Cells and culture medium were recovered at different time points, between 24 and 64 h after transfection, and the amount of rAAV in the samples was determined. Figure 3 shows the kinetics of accumulation of rAAV inside the cells and in the culture medium. The optimal time for harvesting was determined to be between 40 and 48 h after transfection, as the maximal level of rAAV inside the cells had been reached, the ratio pp/Lfu was optimal, and the lowest possible release of rAAV into the medium had taken place. This time interval was chosen for the harvesting of the rAAV produced in the subsequent runs.

The productivity of rAAV obtained by triple transfection was compared for three cell culture systems: triple flasks (7×10^7 cells in two triple flasks per run), two racks-cell factory (1.2×10^8 cells) and ten racks-cell factory (6×10^8 cells). The ratio of transfecting DNA/number of transfected cells was kept constant for all three systems tested. No major difference was observed in the rAAV productivity obtained with each culture system, either measured as pp/cell [8675 ± 350 (flask), 10 350 ± 424 (two racks-cell factory) and 9025 ± 247 (ten racks-cell factory)] or as Lfu/cell [46.4 ± 7.9 (flask), 39.4 ± 6.2 (two racks-cell factory) and 39.5 ± 3.5 (ten racks-cell factory)].

On the basis of the previous results a standard production run (SPR) was defined as consisting of the triple transfection (using pXX6 and pRC4 as helper plasmids) of 293 HEK cells growing in 30 triple flasks with plasmid DNA-PEI complexes, followed by harvesting at 48 h after transfection. Table 2 shows the productivity of rAAV stocks obtained in six independent SPRs. The data in Table 2 indicate that the productivity of our SPR, in addition to being highly reproducible (variation coefficient <9%), is as high as the best result previously reported in the literature using Ad functions as helper for rAAV production [29]. Figure 4 shows the schematic process followed for the rAAV manufacture described in this paper. The process is divided into three main parts: (1) production, (2) downstream processing and (3) characterisation.

The optimised downstream processing consisted of four steps: (1) ultrafiltration-diafiltration through a 300 kDa pore membrane, (2) anionic exchanger column chromatography followed by in-tandem (3) cationic exchanger column chromatography, and finally (4) dialysis against a lactate-Ringer solution.

Figure 5A shows the elution profile obtained following the cationic exchanger column chromatography step. The rAAV peak was contained in the twelve fractions eluting between 28 and 30 mS as shown by analysis of the viral genome, capsid proteins and infectious activity. Figure 5B

Table 2. Reproducibility of PEI-mediated transfection for the production of rAAV

Run #	pp/ml ($\times 10^{13}$)	pp/Lfu	pp/cell
1	2.9	196	9 700
2	3.2	206	12 000
3	3.9	160	12 500
4	3.7	192	11 500
5	2.8	145	12 400
6	3.3	220	11 500
X ± SD	3.3 ± 0.4	186.5 ± 28.5	11 600 ± 1020

rAAV were produced by PEI-mediated triple transfection into two flasks as described in Materials and methods. The rAAV preparations obtained were titered for physical particles (pp) by dot blot assay and for transducing units (Lfu) by X-gal staining as described in Materials and methods.

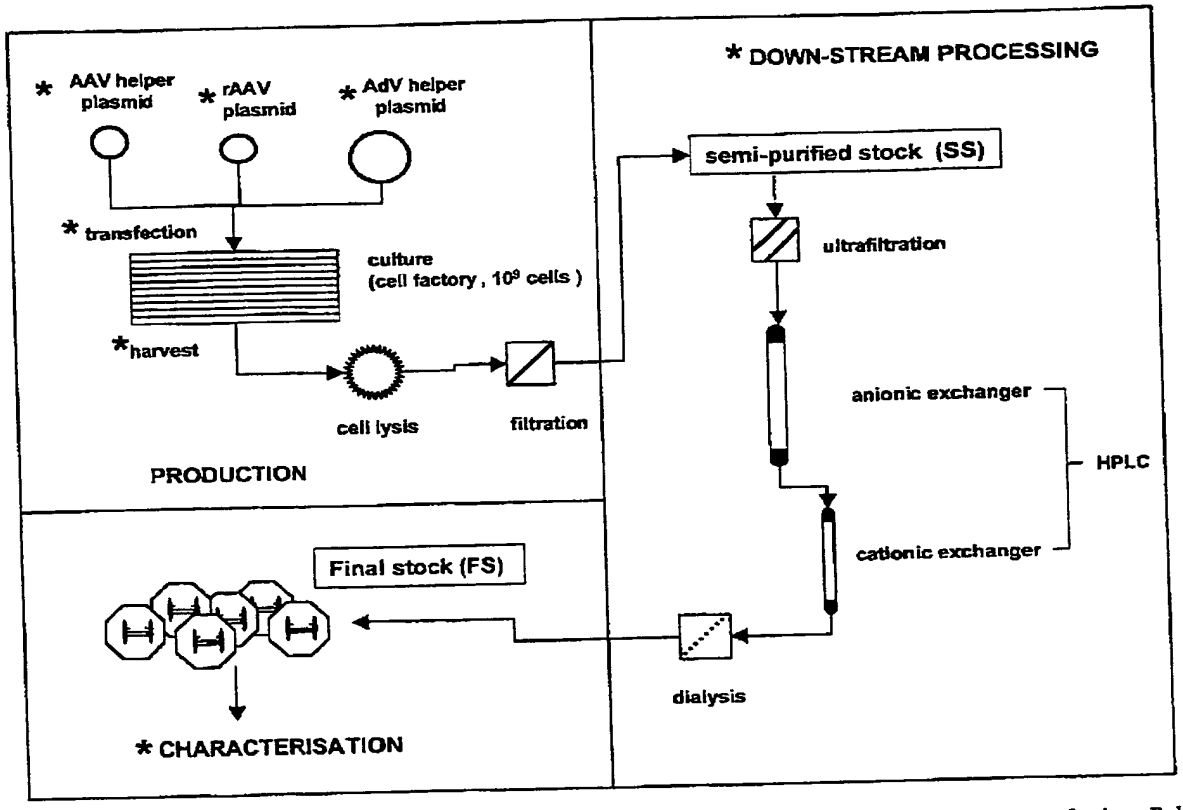


Figure 4. Process for the production and purification of rAAV stocks. rAAV were produced by triple transfection. Relevant downstream process steps included cell lysis, ultrafiltration and HPLC purification. Asterisks indicate those steps that have been optimised throughout the work presented in this paper

and 5C show the elution profile of the rAAV genomes detected by dot blot and rAAV gene transfer activity (Lfu) measured on 293 HEK cells co-infected with Ad (MOI of 5). Figure 5D shows the Western blot analysis for rAAV capsid proteins of the 12 fractions selected. The ratio pp/Lfu obtained in the rAAV peak remains unchanged with respect to those obtained from standard SPR. Fractions 3–7 (see Figure 5B–D) (0.6 ml each) were pooled (3 ml) for further dialysis and characterisation (see below).

Characterisation of rAAV stocks

Protein contamination and content of the rAAV stocks were assessed by SDS-PAGE, ELISA and the Bradford protein assay.

Figure 6 shows the content of total protein and the content of rAAV capsid protein, corresponding to empty plus full (P) and full (pp) rAAV capsids, in SS and FS samples (Figure 6A and 6B, respectively). Figure 6D shows a representative SDS-PAGE gel showing the protein pattern present in rAAV SS (lanes 2 and 3) and FS (lane 4) samples. As shown in the figure, rAAV capsid proteins VP1, VP2 and VP3 are the major individual components of the protein content in the FS pool.

According to the data, 17% of the total protein content in the FS would correspond to full capsids and 33% to empty capsids. Most of the remaining 50% of the FS total protein (e.g. 60 μg protein/ml or 180 μg protein/10^{13} rAAV pp) would probably consist of free rAAV capsid proteins as no other major protein bands are seen in the SDS-PAGE gel (Figure 6D, lane 4). The detection limit of silver staining is 10 ng.

The data in Figure 6C show that our downstream processing leads to a greater than 600-fold decrease in the total protein content between the SS and the FS. Less than 0.2% of the total protein content of a SS was recovered in the FS. As the recovery of rAAV pp from the SS into the FS is ~20% (a decrease of five-fold in the total amount of rAAV pp), then there is an enrichment of more than 100-fold in rAAV ratio pp with respect to the total protein content; 0.22 ± 0.03 and 23.0 ± 1.80 for FS.

The major protein contaminants that might be expected in our rAAV FS samples would be residual adenoviral proteins and β-galactosidase, the transgene in the rAAV vector. In order to confirm the absence of such proteins in the FS pool, Western blot analysis using specific antisera for total Ad proteins, Ad ssDBP and β-gal as well as an enzymatic activity test for β-gal were performed. None of the proteins were detected by Western blots nor by enzymatic assays (data not shown). Based on the detection limit of the Western blot analysis, it can be

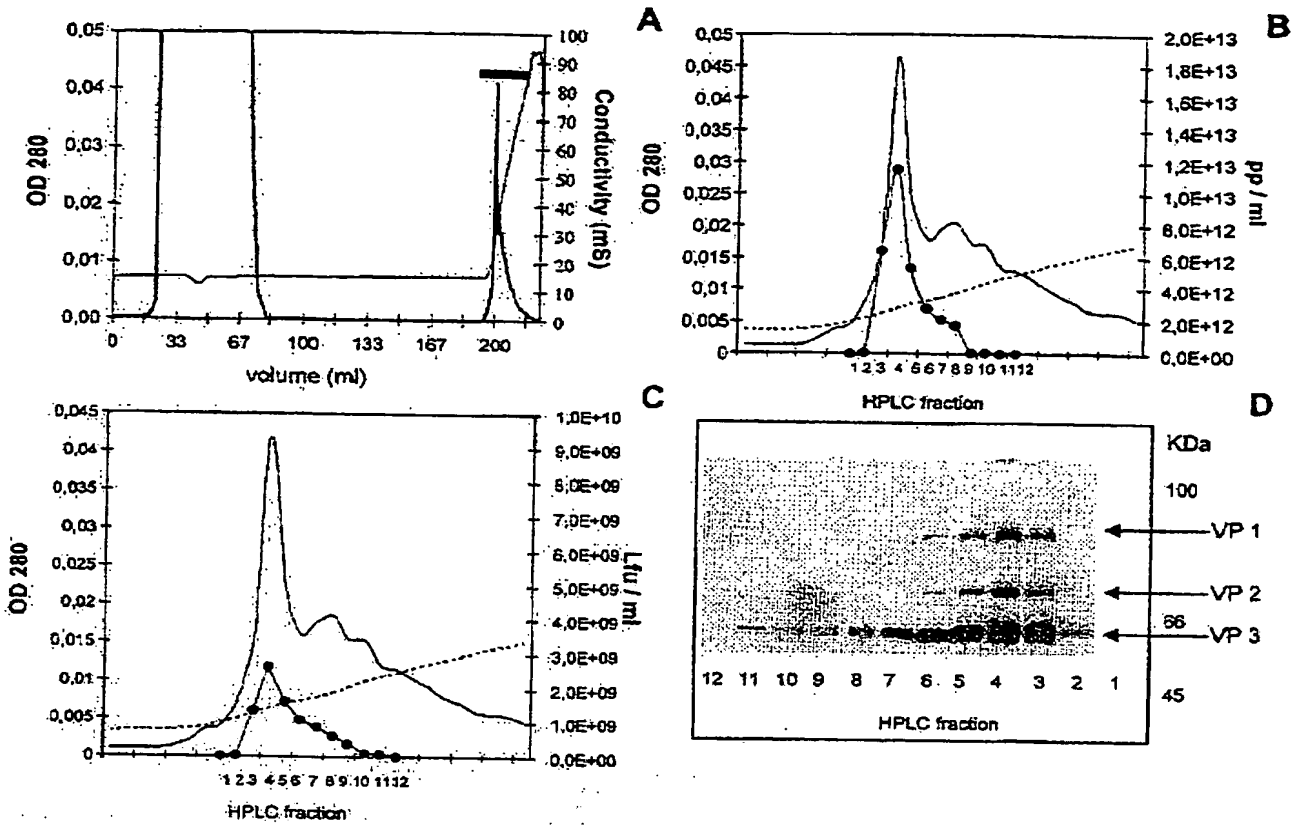


Figure 5. HPLC chromatography of rAAV preparations. rAAV produced as detailed in Figure 4 were purified by a tandem of (anionic followed by cationic) ionic exchange HPLC columns as described in Materials and methods. (A)–(C) Elution profile of the cationic exchange column. (A) Complete elution profile. The thin and thick lines indicate conductivity (mS) and OD_{280} elution profile, respectively. The bar indicates the rAAV eluting at 28–30 mS. (B) and (C) rAAV peak. The broken and solid lines indicate, respectively, conductivity and OD_{280}. Closed circles indicate physical particles (pp) [in (B)] or transducing units (Lfu) [in (C)]. (D) Western blot analysis of the 12 fractions shown with closed circles in (B) and (C), using anti-AAV capsid proteins

concluded that, if there was a contamination with Ad proteins, or with β-gal, it would be less than 5 ng/5×10^{10} rAAV pp each. Based on the detection limit of the β-gal activity assay, it can be concluded that, if there was contamination with β-gal, it would be less than 5 pg/5×10^{10} rAAV pp.

The presence of residual free nucleic acids (RNA, genomic DNA and total dsDNA) contaminating the rAAV FS pool was determined by fluorescent dye binding or by real-time PCR. Table 3 shows the amount of contaminating RNA and total as well as cellular genomic dsDNA present in the rAAV FS and SS. As shown in Table 3, the level of contaminating cellular genomic dsDNA in the rAAV FS is ~35 pg/2–5×10^{12} pp, as quantified by real-time PCR on human repetitive ALU sequences or single copy albumin gene (ALB) sequences, corresponding to a more than 10^5-fold clearance of genomic DNA between the SS and the FS. In contrast, huge amounts of genomic DNA (at least 100 μg/2–5×10^{12} rAAV pp) were obtained when DNA was quantified by ALU-specific real-time PCR on rAAV prepared using an alternative protocol (DNaseI and RNAseA instead of benzonase, and four times $CsCl_2$ gradients instead of HPLC). The difference in the amounts of total and cellular genomic DNA (see Table 3) might be partially due to the presence of residual plasmid DNA in the rAAV FS. According to the data in Table 3, electron micrographs showed the absence of conspicuous DNA molecules in the rAAV FS and significant amounts of residual DNA contaminating the rAAV preparations produced by the alternative $CsCl_2$ gradient protocol. The morphology of the rAAV particles observed in the micrographs was that expected for AAV (data not shown).

The residual amount of benzonase activity present in the rAAV SS and FS is shown in Figure 7. Residual benzonase activity would account for 33 pg protein/ml rAAV FS. The residual amount of benzonase in the FS is <0.0001% of its total protein content (see Figure 6). Most (98.7%) of the benzonase added for the downstream processing is eliminated during HPLC.

Figure 8 shows the expression of the rAAV transgene in mouse muscle *in vivo* after injection of rAAV FS as described in Materials and methods. More than 150 animals were independently injected and analysed using

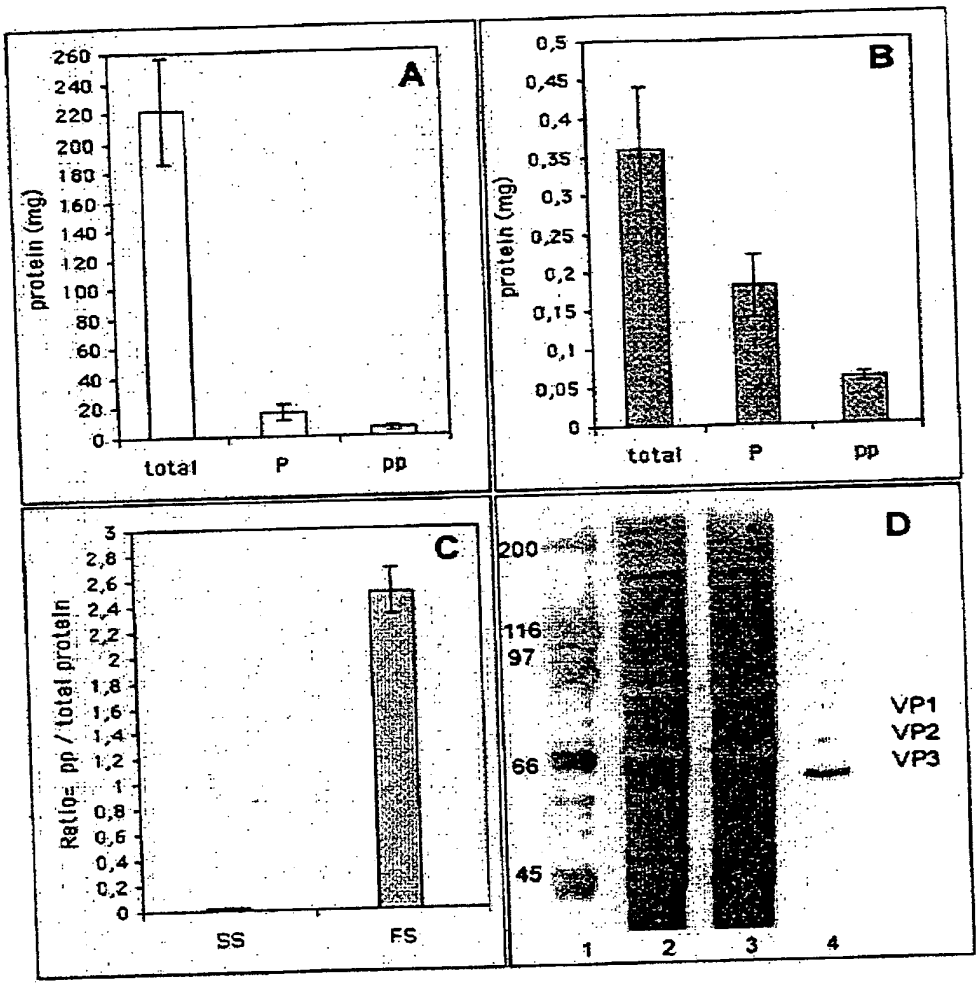


Figure 6. Protein analysis of the rAAV preparations. rAAV were produced and purified as shown in Figure 4. (A) Protein content of the semi-purified stocks (SS). (B) Protein content of the final stocks (FS). Total, content in total protein; P, total content in capsid proteins estimated by ELISA determination; pp, content in rAAV capsid proteins estimated by the number of physical particles. (C) Ratio pp/total protein $\times 10^{12}$ for SS (0.22 ± 0.03) and FS (23.0 ± 1.80). (D) Silver-stained SDS-PAGE gel containing crude lysate (lane 2), SS (lane 3) and FS (lane 4). Molecular weight standards are indicated in lane 1. VP1, VP2, VP3 indicate the expected position of the rAAV capsid proteins

rAAV FS samples (unpublished observations). Consistent data from all 150 animals indicate that our rAAV FS were active *in vivo*, and ensured the transgene expression over a period of at least 35 days after injection. Under the conditions tested, the expression of the lacZ gene largely extended beyond the injection site and, as expected, no signs of cellular immune response in the transduced tissue were observed.

Finally, Table 4 summarises the features of a representative rAAV FS obtained through our optimised production and downstream processes.

Table 3. Residual RNA and DNA contents in rAAV stocks

	Volume (ml)	RNA (ng/ml)	Total dsDNA (ng/ml)	Cellular DNA (ng/ml)
SS	60	5410 ± 1900	1530 ± 110	220 ± 4.10
FS	3	2.00 ± 0.20	0.40 ± 0.06	0.035 ± 0.011
Clearance fold	–	54 000	76 500	125 700

rAAV were produced and purified as shown in Figure 4. Ribo-Green, Pico-Green and real-time PCR (ALU and ALB sequences) detected RNA, total dsDNA and cellular genomic DNA, respectively as described in Materials and methods. SS, rAAV semi-purified stock; FS, AAV final stock ($n=4$).

Discussion

In this paper we describe a process for the production, purification and testing of high-quality, high-purity rAAV prepared in the absence of helper virus.

Production of rAAV in the absence of helper Ad particles has previously been reported [25,26]. In our process, helper Ad functions were provided by expression plasmids carrying the E2, E4 and VA among other Ad

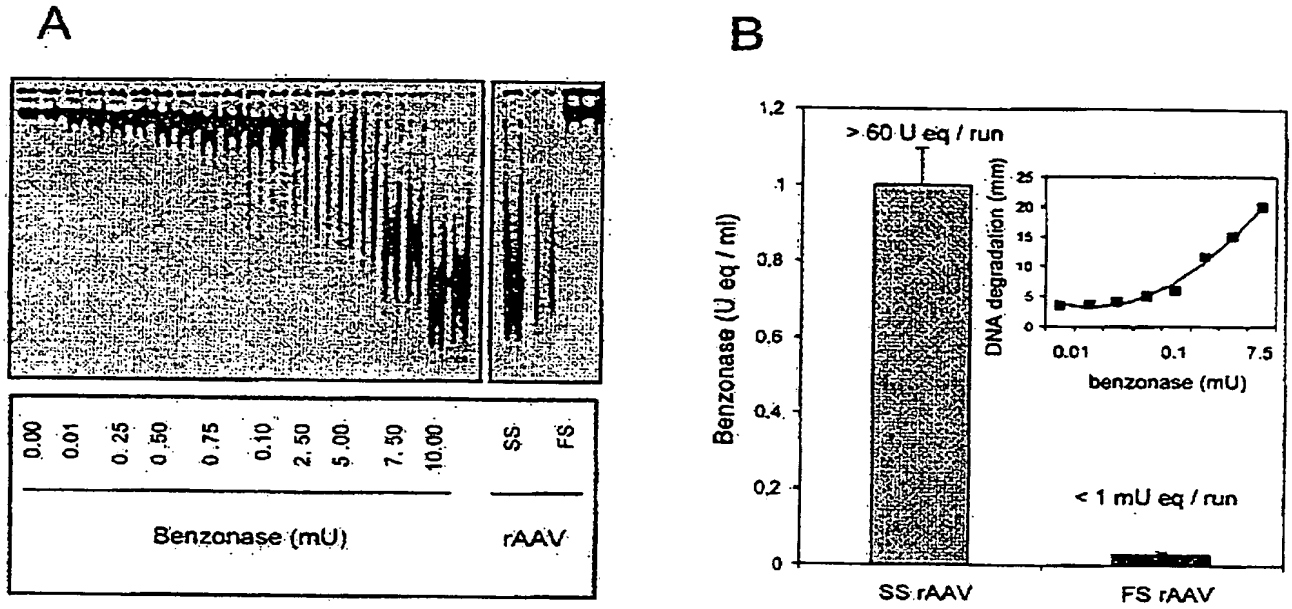


Figure 7. Benzonase residual activity in rAAV stocks. rAAV were produced and purified as shown in Figure 4. Benzonase activity was determined as described in Materials and methods. Ethidium bromide stained agarose gel containing standard plasmid DNA digested either with increasing amounts of a reference benzonase stock or with SS and FS samples (A). The migration of the digested standard DNA as a function of the amount of benzonase present is indicated in (B) (calibration curve). The amounts of benzonase present in the SS and FS are shown in (B) (bars)

genes. Cells were triple transfected with an Ad helper plasmid, an AAV helper plasmid and a rAAV plasmid vector. We demonstrated that PEI and $CaPO_4$ were equally efficient for the transfection-mediated production of rAAV, although PEI produced more reproducible results and lower pp/Lfu ratios. The ease of use of PEI, compared to the $CaPO_4$ precipitation method, together with its higher productivity of infectious particles, makes it the procedure of choice for rAAV manufacturing. Interestingly, large-scale production of rAAV based on PEI-mediated transfection would be more cost-effective than transfection based upon other alternative reagents currently available commercially. In addition, the use of PEI in the transfection step will greatly facilitate further up-scaling of the production process as no culture medium replacement was required following transfection.

The rAAV helper plasmid pRC4, with a deletion of Ad ITR, showed a 1000-fold decrease in the number of contaminant rep+ particles generated during the rAAV production process (Figure 1). According to the literature, the level of contaminating rep+ particles in rAAV stocks varies with the production process itself and with the plasmids used for expression of the rAAV and Ad helper functions [31].

Contamination of rAAV preparations with Ad may lead to unwanted Ad-related immune reactions, unreliable rAAV titer assessment and variable rAAV-mediated gene transfer efficiency. The rAAV preparations obtained using our manufacturing procedure were free of adenovirus particles and proteins as tested by PCR and Western blot (data not shown).

Setting the production process at either the cell factory or triple flask scale level is suitable for obtaining large numbers of rAAV pp sufficient for most *in vivo* evaluations. Toxicology studies in animals are necessary for the further development of rAAV as gene therapy products. In our hands, one run of rAAV production and purification gives $2–5 \times 10^{12}$ pp/ml (i.e. a total of about 1×10^{13} pp). In *in vivo* experiments in mouse muscle, between 40 and 50% of the muscular *tibialis anterior* fibers are transduced when doses of 1×10^{7} ip/animal were used.

It has previously been reported that the efficiency of gene transfer mediated by rAAV vectors is highly dependent on the dose of the rAAV. There is a conspicuous threshold of ip/dose (e.g. 2×10^{6} ip/dose injected into mouse muscle) below which no gene transfer activity can be detected at all (unpublished results). This fact stresses the need for higher titer rAAV preparations and higher doses for most *in vivo* experiments and applications. For evaluation in humans, in phase I trials, the estimated amount of rAAV required is of the order of 2×10^{11} pp/kg [21]. Therefore, an approximate ten times up-scaling of the manufacturing process described in this paper would allow for the production of enough clinical grade material to conduct phase I studies.

Traditionally, two to four centrifugation runs on $CsCl_2$ gradients have purified rAAV. The use of $CsCl_2$ centrifugation would obviously hamper the scaling-up process and probably the release of the clinical material obtained therefrom. In addition, even after four centrifugation runs in $CsCl_2$, the virus obtained was highly contaminated with proteins and DNA (data not shown). Alternatively, the use

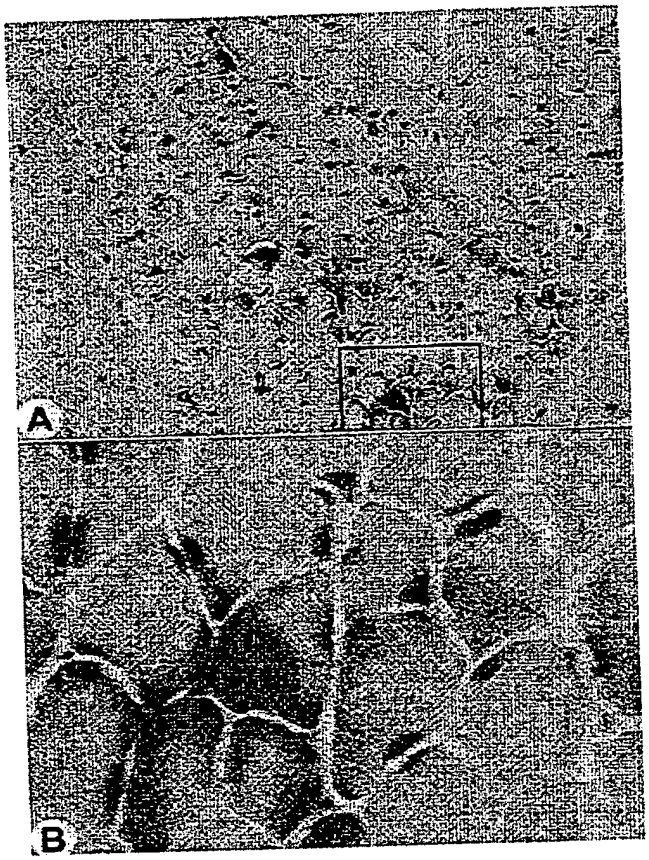


Figure 8. *In vivo* **activity of rAAV stocks in mouse muscle. rAAV were produced and purified as shown in Figure 4. Animals were injected with 1 ± 10^7 ip of rAAV into the *tibialis anterior* and the injected tissue was subsequently analysed as described in Materials and methods. Original magnification: (A) 4× and (B) 10× [insert in (A)]**

of iodixanol-based gradients, although possibly better for obtaining purer rAAV preparations, is probably not up-scalable [35,36]. The use of HPLC chromatography has previously been proposed in place of centrifugation gradients, and a couple of column supports Poros HE/M (heparin) [36,37] and Uno S1 (cation exchanger) [36] have been tested in the literature. In our hands, either heparin-derived supports or (only) cation exchangers gave a poor purification profile leading to rAAV preparations where several (at least 12) major contaminant proteins were still detected on Coomassie blue stained gels. The best results (in terms of infectious particles per milligram of protein) were obtained using a combination of an ultrafiltration/diafiltration step with anionic and cationic exchangers. One additional advantage of our purification system is that no changes of buffer are required between the two columns allowing a simple tandem configuration.

Figure 5 shows that the rAAV can be recovered as a single peak that contains 100% of the pp, Lfu and viral capsid proteins detected. The relatively low efficiency of the downstream processing, which leads to the recovery of only about 20% of the rAAV virus produced, is probably due to the ultrafiltration/diafiltration step (data not shown) which is still to be optimised for higher recovery rates.

rAAV preparations obtained through our production and purification run were characterised by measuring the residual levels of contaminating protein, RNA, DNA, benzonase and rep+/cap+ particles. Table 4 summarises the composition of a routine FS preparation obtained by our protocol which is one of the highest qualities among those previously documented in the literature. In addition, our rAAV stocks have been shown to be active *in vivo* as well as non-immunogenic (no infiltration of the injected muscle with immune cells was observed) (Figure 8).

Benzonase, as well as any other enzyme added at the lysis step, is a potential contaminant in HPLC-purified rAAVs. Therefore they have to be eliminated during the purification steps and their residual activity accurately assessed. In our case, benzonase is the only foreign protein added during downstream processing. Using our purification procedure, as much as 98.7% of the added benzonase was eliminated from the FS. The remaining benzonase activity, which corresponds to 33 pg protein/ml of rAAV preparation, accounts for 0.0001% of the viral protein content of the vector stock.

This is the first report in which rAAV stocks are quantitatively characterised in terms of the content of contaminants (protein, RNA, DNA, genomic DNA, benzonase). The precise characterisation of the rAAV stocks currently used is necessary in order to: (1) validate the stocks with a view to their use in clinical research, (2) generate standard rAAV preparations and comparable results among different laboratories working on basic research as well as preclinical evaluation of rAAV, and (3) help to establish the basis for a results-driven regulation of the clinical development and application of rAAV.

Table 4. Characterisation of a standard rAAV stock

	Quantity/ml	Quantity/run
Physical particles	$2–5 \times 10^{12}$	$6–15 \times 10^{12}$
Infectious particles	$3–6 \times 10^{10}$	$9–18 \times 10^{10}$
pp/ip	35–90	–
pp/cell	9000–12 000	–
rep+ particles	$<10^3$	–
Residual protein	180 µg	–
Residual cellular DNA	<35 pg	–
Residual benzonase	<0.3 mUeq	–

rAAV stocks were produced and purified as described in Figure 4. Characterisation of a standard rAAV stock was performed as described in Materials and methods.

Acknowledgements

The authors thank E. LeCam (Institut G. Roussy, Villejuif, France) for his help with the EM analysis. This work was performed with the financial support of the Association Française contre les Myopathies (AFM).

References

1. Berns KI. Parvoviridae: the virus and their replication. In *Fields in Virology* (3rd edn), vol. 2. Fields BN, Knipe DM, Howley PM (eds). Lippincott-Raven: Philadelphia, 1996; 2173–2197.
2. Miao CH, Snyder RO, Schowalter DB, *et al.* The kinetics of rAAV integration in the liver (Letter). *Nat Genet* 1998; 19: 13–15.
3. Fisher-Adams G, Wong KK, Podsakoff G, Forman SJ, Chatterjee S. Integration of adeno-associated virus vectors in CD34+ human hematopoietic progenitor cells after transduction. *Blood* 1996; 88: 492–504.
4. Wu P, Phillips MI, Bui J, Terwilliger EF. Adeno-associated virus vector-mediated transgene integration into neurons and other non-dividing cell targets. *J Virol* 1998; 72: 5919–5926.
5. Afione SA, Conrad CK, Kearns WG, *et al. In vivo* model of adeno-associated virus vector persistence and rescue. *J Virol* 1996; 70: 3235–3241.
6. Duan D, Sharma P, Yang J, *et al.* Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. *J Virol* 1998; 72: 8569–8577.
7. Vincent-Lacaze N, Snyder RO, Gluzman R, Bohl D, Lagarde C, Danos O. Structure of adeno-associated virus vector DNA following transduction of skeletal muscle. *J Virol* 1999; 73: 1949–1955.
8. Jooss K, Yang Y, Fisher KJ, Wilson JM. Transduction of dendritic cells by DNA viral vectors directs the immune response to transgene products in muscle fibers. *J Virol* 1998; 72: 4212–4223.
9. Kaplitt MG, Leone P, Samulski RJ, *et al.* Long-term gene expression and phenotypic correction using an adeno-associated virus vector in mammalian brain. *Nat Genet* 1994; 8: 148–154.
10. Xiao X, Li J, Samulski RJ. Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. *J Virol* 1996; 70: 8098–8108.
11. Herzog RW, Hagstrom YM, Kung SH, *et al.* Stable gene transfer and expression of human blood coagulation factor IX after intramuscular injection of recombinant adeno-associated virus. *Proc Natl Acad Sci U S A* 1997; 94: 5804–5809.
12. Snyder RO, Spratt SK, Lagarde C, *et al.* Efficient and stable adeno-associated virus-mediated transduction in the skeletal muscle of adult immunocompetent mice. *Hum Gene Ther* 1997; 8: 1891–1900.
13. Goodman S, Xiao X, Donahue RE, *et al.* Recombinant adeno-associated virus-mediated gene transfer into hematopoietic progenitor cells. *Blood* 1994; 84: 1492–1500.
14. Bartlett JS, Samulski RJ, McCown TJ. Selective and rapid uptake of adeno-associated virus type 2 in brain. *Hum Gene Ther* 1998; 9: 1181–1186.
15. Bennett J, Maguire AM, Cideciyan AV, *et al.* Stable transgene expression in rod photoreceptors after recombinant adeno-associated virus-mediated gene transfer to monkey retina. *Proc Natl Acad Sci U S A* 1999; 96: 9920–9925.
16. Dudus L, Anand V, Acland GM, *et al.* Persistent transgene product in retina, optic nerve and brain after intraocular injection of rAAV. *Vision Res* 1999; 39: 2545–2553.
17. Snyder RO, Miao C, Meuse L, *et al.* Correction of hemophilia B in canine and murine models using recombinant adeno-associated-virus vectors. *Nat Med* 1999; 5: 64–70.
18. Greelish JP, Su LT, Lankford EB, *et al.* Stable restoration of sarcoglycan complex in dystrophic muscle perfused with histamine and a recombinant adeno-associated viral vector. *Nat Med* 1999; 5: 439–443.
19. McCown TJ, Xiao X, Li J, *et al.* Differential and persistent expression patterns of CNS gene transfer by adeno-associated virus (AAV) vector. *Brain Res* 1996; 713: 99–107.
20. Flotte T, Carter B, Conrad C, *et al.* A phase I study of an adeno-associated virus-CFTR gene vector in adult CF patients with mild lung disease. *Hum Gene Ther* 1996; 7: 1145–1159.
21. Kay MA, , Manno CS, Ragni MV, *et al.* Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet* 2000; 24: 257–261.
22. Clark KR, Voulgaropoulou F, Johnson PR. A stable cell line carrying adenovirus-inducible rep and cap genes allows for infectivity titration of adeno-associated virus vectors. *Gene Ther* 1996; 3: 1124–1132.
23. Gao GP, Qu G, Faust LZ, *et al.* High-titer adeno-associated viral vectors from a rep/cap cell line and hybrid shuttle virus. *Hum Gene Ther* 1998; 9: 2353–2362.
24. Snyder RO, Xiao X, Samulski RJ. Production of recombinant adeno-associated viral vectors. In *Current Protocols in Human Genetics*, Dracopoli N, Haines J, Krof B, *et al.* (eds). John Wiley & Sons: New York, 1996; 12.1 1–24.
25. Xiao X, Li J, Samulski RJ. Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J Virol* 1998; 72: 2224–2232.
26. Grimm DK, Kern A, Ritter K, Kleinschimdt JA. Novel tools for production and purification of recombinant AAV vectors. *Hum Gene Ther* 1998; 9: 2745–2760.
27. Matsushita T, Elliger S, Elliger C, *et al.* Adeno-associated virus vectors can be efficiently produced without helper virus. *Gene Ther* 1998; 5: 938–945.
28. Conway JE, Rhys CM, Zolotukhin I, *et al.* High-titer recombinant adeno-associated virus production utilizing a recombinant herpes virus type I vector expressing AAV-2 Rep and Cap. *Gene Ther* 1999; 6: 986–993.
29. Snyder RO. Adeno-associated virus-mediated gene delivery. *J Gene Med* 1999; 1: 166–175.
30. Li J, Samulski RJ, Xiao X. Role for highly regulated rep gene expression in adeno-associated virus vector production. *J Virol* 1997; 71: 5236–5243.
31. Salvetti A, Oreve S, Chadeuf G, *et al.* Factors influencing recombinant adeno-associated virus production. *Hum Gene Ther* 1998; 9: 695–706.
32. Drittanti L, Rivet C, Manceau P, Danos O, Vega M. High throughput production, screening and analysis of adeno-associated viral vectors. *Gene Ther* 2000; 7: 924–929.
33. Heid CA, Stevens J, Livak KJ, *et al.* Real time quantitative PCR. *Genome Res* 1996; 6: 986–994.
34. Stanford-Perricaudet LD, Makeh I, Perricaudet M, Briand P. Widespread long-term gene transfer to mouse skeletal muscles and heart via an adenoviral vector. *J Clin Invest* 1992; 90: 626–630.
35. Hermes W, Brake OT, Duzhuizen PA, *et al.* Purification of recombinant adeno-associated virus by iodixanol gradient ultracentrifugation allows rapid and reproducible preparation of vector stocks for gene transfer in the nervous system. *Hum Gene Ther* 1999; 10: 1885–1891.
36. Zolotukhin S, Byrne BJ, Mason E, *et al.* Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gen Ther* 1999; 6: 973–985.
37. Clark RK, Liu X, McGrath JP, Johnson PR. Highly purified recombinant adeno-associated virus vectors are biologically active and free of detectable helper and wild-type viruses. *Hum Gene Ther* 1999; 10: 1031–1039.